STUDIES OF PHOTODYNAMIC ACTION

I. Hemolysis by Previously Irradiated Fluorescein Dyes

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The hemolysis of red blood cells by the combined action of light and certain photoactive substances was first described by Sacharoff and Sachs in 1905. Such hemolysis occurs in a very short time when red blood cells are exposed to sunlight in dilute concentrations of the photoactive substance. Sunlight alone does not produce hemolysis provided the ultra-violet spectrum is screened out by exposing the cells in glass, nor does the photoactive substance in equal concentration in the dark. This is only one of a wide range of similar phenomena brought about under similar conditions in other cells and tissues, which are generally described collectively under the term *photodynamic action* or *photodynamic sensitization*. The photoactive substances which bring these phenomena about include a large number of compounds, most of which are fluorescent dyes. It is generally assumed that such effects are not produced if the solution of the photodynamic substance is separately irradiated, the erythrocytes or other cells being added subsequently in the dark (see Clark 1922, p. 288). There are, however, a few recorded experiments which indicate that this is possible.

Ledoux-Lebard (1902) found that eosine which had been previously exposed to sunlight killed and cytolyzed paramecia; whereas non-irradiated eosine of the same concentration did not. He suggested, therefore, that photodynamic action is due to the formation of a toxic eosine compound by the action of sunlight. Jodlbauer and Tappeiner (1905) found that this did not occur if the eosine solution was neutralized after irradiation and before the addition of the paramecia. They claimed, therefore, that the Ledoux-Lebard effect was due to the formation of acid concomitant with the bleaching of the dye; the acid being the toxic agent. They did not consider this as a photodynamic effect. Sacharoff and Sachs (1905) described hemolysis by previously irradiated β-(o-nitrophenyl)—β-hydroxyethyl methyl ketone [" o-Nitrophenyl-milchsäureketon "]. They were unable to produce hemolysis with previously irradiated eosine or erythrosine, however, and preferred to

[1] Preliminary experiments for these studies were carried out in the Department of Animal Biology, University of Oregon.

consider their one positive result as not belonging to the typical photodynamic phenomena. Fabre and Simonnet (1927) were able to produce hemolysis with lecithin which had been irradiated together with hematoporphyrin by light from a mercury vapour arc. Moore (1928) found that previously irradiated eosine killed the eggs of the sea urchin *Strongylocentrotus purpuratus* but did not cytolyze them; whereas when the eggs were irradiated together with the dye, they were completely cytolyzed. Moore hypothecates the formation of a toxic eosine compound which produces cytolysis upon further irradiation after it has entered the cell. On the other hand, Raab (1900) was unable to produce killing of paramecia by previously irradiated acridine solutions. Hausmann was unable to produce killing of paramecia or hemolysis with previously irradiated solutions of chlorophyll (1909) or hematoporphyrin (1910); although similar solutions produced these effects when irradiated together with the cells. Hasselbach (1909) could not produce hemolysis with previously irradiated solutions of several photodynamically active substances including eosine and erythrosine. Pereira (1925) found that *Arbacia* larvæ were not killed by previously irradiated eosine in sea water.

The writer has found that it is possible, under carefully controlled conditions, to bring about hemolysis with previously irradiated solutions of the three fluorescein dyes which he has investigated, fluorescein, eosine and erythrosine. This is of considerable interest because of its bearing on certain theories of photodynamic action which will be discussed later in this paper.

Experimental

Hemolysis by previously irradiated fluorescein, eosine, and erythrosine.—The writer's first attempts to produce hemolysis with previously irradiated eosine solutions met with apparent success in only a few instances. These were thought at first to be accidental, but with more careful control of conditions it was found possible to obtain consistently reproducible results. The successful technique required the selection of proper hydrogen ion concentration and dye concentration.

The hydrogen ion concentration must be carefully buffered, since unbuffered solutions tend to increase in acidity during irradiation. This increase in acidity may inhibit the production of hemolysis by bringing about fixation of the cells as will be pointed out in a later paper. To insure the maximum obtainable buffering capacity, it was found convenient to make up the dye in solutions of primary and secondary sodium phosphate mixtures. In order to insure a medium of proper osmotic pressure for the blood cells, the phosphate mixtures were

calculated to have the same osmotic pressure as a 0.15 M sodium chloride solution. This was done by assuming that the primary phosphate dissociates into two ions, the secondary phosphate into three. The mol fractions of the two salts required for a given hydrogen ion concentration were estimated by the use of Cohn's data for potassium phosphates (see Clark, 1928, pp. 216–220).[2] The hydrogen ion concentrations of the solutions were checked by means of the hydrogen electrode. Such solutions proved rather unsatisfactory in the case of fluorescein, and a solution containing 10 per cent of the phosphate mixture and 90 per cent 0.15 M sodium chloride, was used instead in most experiments with this dye. The concentration of phosphate in this solution is still many times that of fluorescein in most of the dye concentrations which were used, and affords an adequate buffer.

The optimal concentration of the dye varies with a number of conditions; some of which, as for example the intensity of irradiation, it was impossible to control. It was found expedient, therefore, to use a series of dilutions of the dyes; usually consisting of ten dilutions from 1 per cent to 0.002 per cent.[3] These were exposed to the sunlight for a given period of time. Blood cells were then added to the irradiated solutions and also to a control consisting of a corresponding series of non-irradiated dye solutions. Both series were then placed in a dark room where the temperature was in the region of 20° C. Observation of the tubes for hemolysis was made at intervals after the addition of the cells. It was found that in most cases six hours sufficed for the hemolysis to reach a maximum. Since the temperature during irradiation could not be controlled, time was allowed, when necessary, for the irradiated tubes to come to the same temperature as the controls before adding the cells. The solutions were exposed in small test tubes (10 x 75 mm.), each containing 2 cc. of the solutions. The blood cells were added to each tube in the quantity of 0.02 cc. of a 50 per cent suspension in 0.15 M sodium chloride, by means of a blood pipette. This method avoids any appreciable dilution of the irradiated solution upon the addition of the cells. This precaution has not been observed by most of the investigators who have attempted to produce hemolysis with previously irradiated substances. Human blood cells were used

[2] Dr. G. Payling Wright and the writer have found that rabbit blood cells suspended in such solutions show a variation in volume of approximately twelve per cent over the range of hydrogen ion concentration between pH 7.7 and pH 6.0, and have approximately the same volume as cells in serum.

[3] The dyes used were Fluorescein, sodium salt (Uranine), from the National Aniline and Chemical Company, Erythrosine B (sodium salt of tetra-iodo-fluorescein) also from the National Aniline and Chemical Company, and Eosine Y (sodium salt of tetra-brom-fluorescein) from Coleman and Bell.

in most of the experiments. They were washed by centrifuging three times from suspension in 0.15 M sodium chloride to free them from serum. It is advisable to have the cells as free from serum as possible, since serum is effective in preventing photodynamic hemolysis (Busck, 1906). The intensity of the radiation could not be accurately estimated, but it was found practicable to expose the solutions to bright midday sunlight for one to two hours. Too long continued exposure causes bleaching of the dye, resulting in a lowered concentration of the active dye.

TABLE I

Hemolysis by Previously Irradiated Fluorescein

Solutions exposed to sunlight 90 minutes (2:00–3:30 P.M., September 29, 1929). All solutions contain 10 per cent of sodium phosphate buffer, pH 6.4, isosmotic with 0.15 M NaCl, plus 90 per cent of 0.15 M NaCl. Observations made after 16 hours in dark following addition of red blood cells. *H* = complete hemolysis, (*H*) = partial hemolysis, and the dash is used when there is no detectable hemolysis.

Concentration of fluorescein	Irradiated solution. Red blood cells added after		Non-irradiated solution (control)
per cent	45 minutes	4 hours	
1.0	—	—	—
0.5	—	—	—
0.25	—	—	—
0.125	(H)	—	—
0.062	(H)	(H)	—
0.031	H	(H)	—
0.015	H	H	—
0.007	(H)	(H)	—
0.004	(H)	(H)	—
0.002	—	—	—
0.00	—	—	—

Tables I, II, and III show the results of typical experiments with fluorescein, eosine and erythrosine respectively. In these tables, *H* represents complete hemolysis (*i.e.* hemochromolysis and stromatolysis) as well as can be judged by the naked eye, (*H*) represents partial hemolysis, and the dash no detectable hemolysis. These classifications are arbitrary, but since comparison can always be made with the control tubes, there can be no doubt of the general validity of the observations. An examination of Tables I, II, and III demonstrates quite clearly that previously irradiated solutions of these dyes bring about hemolysis in concentrations at which non-irradiated solutions do not. Some bleaching of the dye takes place upon irradiation and this raises the question whether the hemolysis may not be due to the products of this bleaching.

It has been found, however, that completely bleached solutions have no hemolytic action.

Non-irradiated eosine and erythrosine produce hemolysis in sufficiently high concentration, as is shown in Tables II and III. This was described by Sacharoff and Sachs (1905) and studied by Tappeiner (1908). It is apparently not due to irradiation during the preparation of the solutions; since in these experiments the results were the same when the solutions were carefully prepared in the dark room under red light, which is outside the absorption range of these dyes, as when

TABLE II

Hemolysis by Previously Irradiated Eosine

Solutions exposed to sunlight for 105 minutes (11:45A.M.–1:30 P.M., September 10, 1929). All solutions contain sodium phosphate buffer, pH 7.0, isosmotic with 0.15 M NaCl. Observations made 5 hours after addition of red blood cells. The symbols are the same as those in Table I.

Concentration of eosine	Irradiated solution. Red blood cells added after			Non-irradiated solution (control). Red blood cells added after	
per cent	45 minutes	2¾ hours	5 hours	45 minutes	5 hours
1.0	H	H	H	H	H
0.5	H	H	H	(H)	(H)
0.25	H	H	H	—	—
0.125	H	H	H	—	—
0.062	H	H	H	—	—
0.031	H	(H)	H	—	—
0.015	(H)	(H)	(H)	—	—
0.007	—	—	—	—	—
0.004	—	—	—	—	—
0.002	—	—	—	—	—
0.0	—	—	—	—	—

prepared with ordinary precautions in the diffuse light of the laboratory. The effect of short exposure to diffuse light is thus within the accuracy of the observations described here. The absence of hemolysis in the higher concentrations of the irradiated dye in Table I is probably due to fixation of the cells. This phenomenon will be discussed in a later paper. The marked effect of hydrogen ion concentration upon the hemolytic activity of irradiated and non-irradiated dyes will also be discussed in that paper; the hydrogen ion concentrations for the experiments here described have been chosen as those at which the difference in hemolytic activity between previously irradiated and non-irradiated solutions could be most clearly demonstrated.

It will be noted in Tables I, II, and III that the results are changed

very little when the exposed solutions are allowed to remain in the dark for as much as four or five hours after irradiation, before the addition of the cells. This shows very conclusively that the increased hemolytic activity of the irradiated solutions cannot be due to their having a greater temperature than the controls because of the absorption of heat during the period of exposure, since ample time is allowed for the two series of solutions to come to the same temperature. It also demonstrates that whatever change occurs in the course of irradiation is not rapidly reversible in the dark. Moore (1928) observed, similarly,

Table III

Hemolysis by Previously Irradiated Erythrosine

Solutions exposed to sunlight for one hour (11:00 A.M.–12:00 M., September 28, 1929). All solutions contain sodium phosphate buffer, pH 6.5, isosmotic with 0.15 M NaCl. Observations made 6 hours after addition of red blood cells. The symbols are the same as those in Tables I and II.

Concentration of erythrosine	Irradiated solution. Red blood cells added after			Non-irradiated solution (control)
per cent	45 minutes	1¾ hours	5 hours	
1.0	H	H	H	H
0.5	H	H	H	H
0.25	H	H	H	H
0.125	H	H	H	H
0.062	H	H	H	(H)
0.031	H	H	H	(H)
0.015	(H)	(H)	(H)	—
0.007	—	—	—	—
0.004	—	—	—	—
0.002	—	—	—	—
0.00	—	—	—	—

that in the case of the killing of sea urchin's eggs by previously irradiated eosine, the solution retained its toxic properties after six hours in the dark.

Discussion

Numerous hypotheses have been developed to explain the mechanism of photodynamic action, most of which contain the assumption that the photodynamic substance and substrate (*e.g.* cells) must be irradiated together. This is true of the theory of Tappeiner (1909) which he outlines as follows: The presence of the photodynamic substance merely accelerates the action of visible light. The split products of this reaction are removed through oxidation by molecular oxygen. Ordinarily

these products accumulate and inhibit the reaction, but the combined action of light and a photodynamic substance accelerates their removal and consequently the total reaction. Another conception, based on the fact that most of the photodynamic substances are fluorescent, is that the photodynamic effects are due to the action of fluoresced radiation upon the protoplasm. Since the fluoresced light is only a more or less polarized radiation from a particular region of the visible spectrum characteristic of the substance concerned (Pringshein 1928, p. 195), it can hardly be expected to have such destructive effects. Moreover, Raab (1900) showed that paramecia are not damaged when exposed to the fluoresced radiation from a solution of fluorescent substance with which they are not in contact; and likewise, Sacharoff and Sachs (1905) showed that red blood cells exposed under the same conditions are not hemolyzed. Nevertheless, this concept remains current to a certain extent. Schanz (1921) suggests from studies on the photoelectric effect in albumin, and albumin plus fluorescein dyes, that the changes brought about in the cell constituents are due to the absorption of electrons emitted by the dye during irradiation. Clark (1922, pp. 302–303) suggests that the photodynamic substance shifts the photoelectric threshold of the cell constituents from the ultra-violet into longer wave lengths. Metzner (1924) claims that the photodynamic effects are brought about by an action within the cell dependent upon the combination (adsorption) of the dye with the protoplasm. Jodlbauer (1926) assumes that the dye must be adsorbed by the cell, and that only those dyes are photodynamically active which retain their ability to be activated by light while in combination with the cell substance. Such theories demonstrate how firmly the idea is established that the photodynamic substance and substrate must be irradiated together. Obviously all such explanations of photodynamic action must be discarded or modified, in light of the fact that hemolysis may be brought about by previously irradiated photodynamic substances.

Experimental

Evidence that Oxidation Is a Factor in Photodynamic Hemolysis.— A theory of direct oxidation of cell constituents by the action of light and the photodynamic substance was put forward by Straub (1904*a*). His hypothesis was founded principally upon the analogy between the photodynamic action of eosine upon cells and its ability to oxidize iodide ion in the presence of light. He found (1904*b*) that, in proper concentration, eosine may oxidize many times its equivalency of iodide when the two substances are exposed to sunlight together in solution. He conceived that the eosine is changed to an eosine peroxide by the action

of light; and that this peroxide brings about the oxidation of an equivalent amount of iodide ion, being returned in so doing to the original eosine form. The eosine may then proceed to the oxidation of another quantity of iodide, thus acting in a sense as a catalyst. He could not, however, demonstrate the existence of an intermediate peroxide, being unable to obtain conclusive evidence of the oxidation of iodide ion by the action of previously irradiated eosine (1904*a*).

The writer finds that previously irradiated eosine will oxidize iodide ion, as shown by a positive starch reaction after adding potassium iodide in the dark. The oxidation proceeds rather slowly immediately after the addition of the potassium iodide, which may account for Straub's failure to observe it in his experiments. Table IV presents some quantitative results obtained when (1) fluorescein dyes and potassium iodide were irradiated in solution together, and (2) when the iodide was added to the previously irradiated dyes. The determinations were made by titration of the free iodine formed due to the oxidation of iodide ion, with 0.001 N sodium thiosulfate against starch indicator. When potassium iodide is added to the previously irradiated dye and the mixture placed in the dark, the oxidation takes place quite slowly, reaching a maximum after about three hours. The titrations were, therefore, performed after the elapse of this time. The accuracy of determination of iodine in such small concentrations is, of course, subject to some error. In order to determine the magnitude of this error, solutions containing quantities of iodine of the same order as those represented in Table IV were titrated. The solutions were of the same volume, contained the same concentration of dye and of potassium iodide, and were buffered at the same hydrogen ion concentration as the experimental solutions. With concentrations of iodine corresponding to the lowest values in Table IV, the determinations were consistently 10 to 15 per cent lower than the theoretical. With quantities of iodine corresponding to the highest values the error was not greater than one per cent. The 0.001 N thiosulfate solution was always freshly prepared by dilution from a 0.1 N stock solution.

The experiments described in Table IV represent conditions in the region of the optimal for the reaction of the iodide with each dye. The extent of these reactions seems to be greatly affected by the hydrogen ion concentration, and by other factors, which will not be discussed here. Controls containing the same concentration of potassium iodide, but no dye, never showed more than a trace of free iodine when exposed to sunlight simultaneously with the potassium iodide-dye mixtures. Likewise, solutions of the dye containing potassium iodide showed no trace of free iodine after many hours in the dark.

Table IV

Oxidation of Potassium Iodide by Irradiated Fluorescein, Eosine, and Erythrosine

Fluorescein	KI	pH	Volume of Solution	Duration of Irradiation	KI Added after Irradiation	Volume of $Na_2S_2O_3$ 0.001N	Mols of Dye	Mols of Iodide Oxidized
	per cent		*Cc.*	Hours	*per cent*	*Cc.*		
0.0005M	1.0	6.0	6.0	8	0.0	18.6	3×10^{-6}	18.6×10^{-6}
0.0005M	1.0	6.0	6.0	8	0.0	17.1 *	3×10^{-6}	17.1×10^{-6}
0.0005M	1.0	6.0	6.0	0	0.0	0.0	3×10^{-6}	0.0
0.0005M	0.0	6.0	6.0	8	3.0	1.0 †	3×10^{-6}	1.0×10^{-6}
0.0005M	0.0	6.0	6.0	8	3.0	0.5 ‡	3×10^{-6}	0.5×10^{-6}
0.0	1.0	6.0	6.0	8	0.0	0.4	0.0	0.4×10^{-6}
Eosine								
	per cent		*Cc.*	Hours	*per cent*	*Cc.*		
0.001M	3.0	6.0	6.0	6	0.0	14.5	6×10^{-6}	14.5×10^{-6}
0.001M	3.0	6.0	6.0	6	0.0	14.5 *	6×10^{-6}	14.5×10^{-6}
0.001M	3.0	6.0	6.0	0	0.0	0.0	6×10^{-6}	0.0
0.001M	0.0	6.0	6.0	6	3.0	3.2 †	6×10^{-6}	3.2×10^{-6}
0.001M	0.0	6.0	6.0	6	3.0	3.0 ‡	6×10^{-6}	3.0×10^{-6}
0.001M	0.0	6.0	6.0	0	3.0	0.0	6×10^{-6}	0.0
Erythrosine								
	per cent		*Cc.*	Hours	*per cent*	*Cc.*		
0.001M	3.0	6.0	6.0	6	0.0	19.1	6×10^{-6}	19.1×10^{-6}
0.001M	3.0	6.0	6.0	6	0.0	19.5 *	6×10^{-6}	19.5×10^{-6}
0.001M	3.0	6.0	6.0	0	0.0	0.0	6×10^{-6}	0.0
0.001M	0.0	6.0	6.0	6	3.0	1.3 †	6×10^{-6}	1.3×10^{-6}
0.001M	0.0	6.0	6.0	6	3.0	1.0 ‡	6×10^{-6}	1.0×10^{-6}
0.001M	0.0	6.0	6.0	0	3.0	0.0	6×10^{-6}	0.0

* Titration after 3 hours in dark.
† KI added immediately after irradiation with titration after 3 hours in dark.
‡ KI added after 3 hours in dark following irradiation; titration 3 hours later.

Table IV shows that iodide ion equivalent to several times the quantity of dye present may be oxidized when exposed together with the dye (equivalency considered as one mol of iodide ion per mol of dye). Straub (1904*b*) was able, in fact, to oxidize a quantity of iodide ion sixty-five times as great as the quantity of dye present. On the other hand, when the dye alone is irradiated and the potassium iodide added subsequently in the dark, the quantity of iodide ion oxidized is always less than that equivalent to the dye present. In the latter case it was never found possible, in a considerable number of experiments under varying conditions, to oxidize more iodide than a quantity equivalent

to the quantity of dye present. When irradiated in the absence of a readily oxidizable substance, such as iodide ion, a certain amount of the dye is oxidized, as is indicated by bleaching. Thus the transformation of all the dye to the active form cannot be expected, and we should expect that less iodide would be oxidized than a quantity equivalent to the quantity of the dye originally present. This appears to be the case. When the dye is exposed with a readily oxidizable substance, no bleaching occurs, indicating that this substance is oxidized instead of the dye. All these facts lend support to Straub's hypothesis. They demonstrate at least that a substance is formed upon irradiation of the dye solution which is capable of oxidizing substances which the non-irradiated dye cannot, and indicate that this is an intermediate substance in the oxidations brought about by the action of the dye and light.

The quantity of iodide ion oxidized is not greatly altered if the dye is allowed to remain in the dark for several hours after irradiation before potassium iodide is added. This shows that the change brought about by irradiation is not rapidly reversible in the dark. This is exactly parallel to the case of hemolysis where, as we have seen, hemolysis is brought about by previously irradiated dye solutions which have remained in the dark for several hours after irradiation before addition of the cells. Substances produced in the bleaching of the dye are not responsible for the oxidation of iodide ion, since completely bleached solutions do not bring about this oxidation. This is again parallel to the case of hemolysis, since as stated above, hemolysis is not produced by completely bleached dyes. These latter facts suggest very definitely that the substance in irradiated solutions of a fluorescein dye which brings about hemolysis is the same as that which brings about the oxidation of iodide ion; and that, therefore, the former process is probably dependent upon an oxidation.

If it is true that the hemolysis of blood cells by irradiated dyes involves the oxidation of cell constituents in a manner similar to the oxidation of iodide ion, we should expect, parallel to the above observations, more extensive oxidation and thus greater hemolysis when the dye is irradiated together with the cells than when previously irradiated. In the former case the dye may, presumably, act in a catalytic sense, thus oxidizing several times its molecular equivalency of cell constituents; whereas in the latter case the amount of oxidation is limited by the quantity of dye present. The data presented in Tables V, VI and VII, appears to confirm this prediction; the hemolytic action seems to be quantitatively much greater when the dye and cells are irradiated together than when the dye is irradiated alone and the cells added later in the dark. The statement that hemolysis is more readily produced

Table V

Comparison of Hemolytic Activity of Fluorescein Irradiated With and Without Blood Cells

Solutions exposed to sunlight for one hour and 30 minutes. All solutions contain sodium phosphate buffer, pH 6.5, isosmotic with 0.15 M NaCl. Observations made after 20 hours in dark following addition of blood cells. Symbols as in preceding tables. *P* = precipitate.

Concentration of Fluorescein	Fluorescein Solution Irradiated with Cells	Fluorescein Irradiated Alone. Cells Added in Dark	Fluorescein Not Irradiated
per cent			
1.0	P	(H)	—
0.5	P	(H)	—
0.25	P	(H)	—
0.125	H	(H)	—
0.062	H	(H)	—
0.031	H	(H)	—
0.015	H	(H)	—
0.007	H	—	—
0.004	H	—	—
0.002	H	—	—
0.0	—	—	—

when the dye is irradiated together with the cells than when irradiated separately is a generalization to which many exceptions occur, due chiefly to the complicating factor of fixation which will be considered in a later paper. That hemolysis may proceed farther in the former case than in the latter, in conditions where fixation is not a complicating

Table VI

Comparison of Hemolytic Activity of Eosine Irradiated With and Without Blood Cells

Solutions exposed to sunligh tfor one hour and 30 minutes. All solutions contain sodium phosphate buffer, pH 6.9, isosmotic with 0.15 M NaCl. Observations made after 7 hours in dark following irradiation. Symbols as in the preceding tables.

Concentration of Eosine	Eosine Solution Irradiated with Cells	Eosine Irradiated Alone. Cells Added in Dark	Eosine Not Irradiated
per cent			
1.0	H	H	H
0.5	(H)	H	(H)
0.25	H	H	—
0.125	H	H	—
0.062	H	H	—
0.031	H	H	—
0.015	H	(H)	—
0.007	H	—	—
0.004	H	—	—
0.002	H	—	—
0.0	—	—	—

factor, seems justified by all the writer's observations on red blood cells under the two conditions. It would be, of course, absurd to attempt an exact quantitative comparison between the results in Table IV and those in Tables V, VI, and VII, since we do not know in the case of the blood cells, what substances may be subject to oxidation, or what their oxidation-reduction potentials may be.

The action of non-irradiated dyes, previously mentioned, is in all probability not an oxidative process, since oxidation of iodide ion by these dyes does not take place in the dark. Whatever the nature of this process, however, when hemolysis occurs after irradiation in a concentration of dye which does not produce hemolysis when not irradiated, we are justified in the assumption that the changes bringing about hemolysis may be oxidative, since we know that the oxidizing power of the dye solution has been increased by irradiation.

Table VII

Comparison of Hemolytic Activity of Erythrosine Irradiated With and Without Blood Cells

Solutions exposed to sunlight for one hour. All solutions contain sodium phosphate buffer, pH 7.0, isosmotic with 0.15 M NaCl. Observations made after 6 hours and 20 minutes in dark following irradiation. Symbols as in preceding tables.

Concentration of Erythrosine	Erythrosine Solution Irradiated with Cells	Erythrosine Irradiated Alone. Cells Added in Dark	Erythrosine Not Irradiated
per cent			
1.0	H	H	H
0.5	H	H	H
0.25	H	H	H
0.125	H	H	H
0.062	H	H	H
0.031	H	H	—
0.015	H	(H)	—
0.007	H	—	—
0.004	H	—	—
0.002	H	—	—
0.0	—	—	—

Discussion

Further evidence that oxidation is an important factor in photodynamic processes is not lacking. Oxygen is known to be necessary for a number of photodynamic effects (Straub, 1904*a*; Jodlbauer and Tappeiner, 1905), since they do not take place in its absence. Specifically as regards hemolysis, Hasselbach (1909) found that hemolysis by light and certain photodynamic substances, including eosine and

erythrosine, did not take place in a vacuum, and Schmidt and Norman (1922) found that hemolysis by eosine and sunlight did not occur in hydrogen. Sacharoff and Sachs (1905) showed that the presence of the reducing substance *sodium sulfate* may prevent hemolysis by irradiated erythrosine. Noack (1920) showed that a number of inorganic reducing agents may inhibit photodynamic effects, and Schmidt and Norman (1922) found that a number of readily oxidizable organic and inorganic substances will prevent hemolysis by eosine and light. Noack (1920) has also shown quite definitely that certain plant pigments can be oxidized by various photodynamic substances and light, and gives evidence that these phenomena involve the formation of intermediate peroxides.

Conclusions

The demonstration of the formation of an intermediate substance in the process of photodynamic hemolysis by fluorescein dyes offers quite conclusive evidence against the sensitization theory of Tappeiner and other theories which assume that photodynamic substance and substrate must be irradiated together. The demonstration that a definite increase in the oxidizing power of solutions of these dyes is brought about by irradiation, together with the accumulation of other evidence pointing toward an oxidative process, makes it necessary to consider the oxidation of cell constituents as a probable underlying factor in photodynamic hemolysis. Likewise, such oxidations must be considered as a possible factor in all photodynamic processes.

Summary

1. Hemolysis may be produced by previously irradiated fluorescein, eosine and erythrosine.

2. Similarly, previously irradiated fluorescein, eosine and erythrosine oxidize iodide ion.

3. These findings render untenable the sensitization theory of Tappeiner and other theories which necessitate the simultaneous action of light and the photodynamic substance, while supporting Straub's theory of direct oxidation of cell constituents.

4. Oxidation must be considered as a probable underlying cause in photodynamic hemolysis and all other photodynamic phenomena.

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